Human Erythropoietin-Specific Sites of Monoclonal Antibody-Mediated Neutralization


Recombinant human erythropoietin (rhuEpo)-specific mouse monoclonal antibodies (MoAbs) have been produced and characterized. All antibodies were specifically reactive with rhuEpo in enzyme-linked immunosorbent assay (ELISA). Epitope exclusion studies showed three distinct epitope regions, A, B, and C, recognized by neutralizing MoAbs. An additional epitope region D was recognized by non-neutralizing MoAbs. Antibodies defining an epitope region competed with each other for binding sites, but did not compete with antibodies defining a different epitope region. Group B antibodies were able to compete for the receptor binding site on rhuEpo with a soluble human Epo-receptor-Ig fusion protein. No single peptide sequences were found to specifically interact either with group B MoAbs or with the rhuEpo-receptor. Therefore, it is suggested that epitope region B and the receptor binding site share binding determinants that are primarily composed of conformational epitopes. Because group A and group C antibodies did not compete with the receptor for binding to the receptor binding site of the rhuEpo molecule, it is suggested that neutralization via epitope regions A and C is mediated through binding inhibition caused by conformational changes, transmuting the binding site(s) for the receptor. Conversely, binding to the receptor seems to induce conformational changes in the hormone molecule, eliminating epitopes for group A and C antibodies.

To further identify relevant functional sites of the huEpo molecule we searched for neutralizing mouse MoAbs competing for the receptor binding site. For immunization we used the whole rhuEpo molecule or the C-terminal peptide P2.7

MATERIALS AND METHODS

Immunization. Female Balb/c mice received subcutaneous or intraperitoneal injections of 20 µg rhuEpo, rhuEpo-keyhole limpet hemocyanine (KLH), or synthetic peptide-KLH respectively emulsified in complete Freund's adjuvant followed by three to four additional immunizations with incomplete Freund's adjuvant every 4 weeks. The last 4 days before fusion mice were boosted intravenously (10 µg/day).

Development and screening of MoAbs. Spleen cells were isolated aseptically and were fused with the myeloma cell line SP 2/0 using polyethylene glycol(PEG) according to standard methods. The final cell pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal calf serum (FCS) and HAT (0.1 mmol/L hypoxanthin, 0.004 mmol/L aminopterin, and 0.016 mmol/L thymidine) and added to 24-well culture plates (Nunc GmbH, Wiesbaden, Germany). About 14 days later, individual cell clones were picked out and transferred to a new well. Three days later, supernatants were tested for antibody content as well as for the presence of Epo-specific antibodies. Positive cell cultures were grown up and frozen in liquid nitrogen. In parallel, positive cell lines were cloned using a single cell manipulator.

The enzyme immunoassays (EIA) used for screening the presence of anti-rhuEpo antibodies in cell culture supernatants were carried out as follows. Each well of a microtitation plate (Nunc) coated with rabbit anti-mouse Ig (Behringwerke AG, Marburg, Germany) was filled with 100 µL of supernatant and incubated for 1 hour at room temperature. After washing 100 µL of a rabbit anti-mouse-POD conjugate were added for an additional hour at room temperature. Following a 30 minute incubation with substrate color development was read at 492 nm on a Behring ELISA Processor (Behringwerke AG).

In parallel culture, supernatants were tested in a second screening test using rhuEpo-coated microtitation plates (Nunc GmbH). After washing 100 µL of a rhuEpo-peroxidase (POD)-conjugate were added for an additional hour at room temperature. Following a 30 minute incubation with substrate color development was read at 492 nm on a Behring ELISA Processor (Behringwerke AG).

Production and purification of MoAbs. Cell lines producing anti-Epo MoAbs were propagated in mass culture. IgGs purified by affinity chromatography using protein A-sepharose (Pharmacia, Uppsala, Sweden) according to the manufacturer. Purity was monitored by high performance liquid chromatography (HPLC) and so-

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Preparation of a soluble rhuEpo-receptor Ig fusion protein. The construction of an expression plasmid encoding an Ig fusion protein and its characterization will be described in detail elsewhere (M.R. Fibi et al, manuscript in preparation). Brieﬂy, cDNA encoding the complete extracellular region of the huEpo receptor was polymerase chain reaction (PCR) ampliﬁed from plasmid pLAP37, which we received as a kind gift from J. G. Winkelmann. Primers were designed to allow the creation of a unique HindIII site in the 5' untranslated region and a BamHI site at the end of the region encoding the extracellular portion of the huEpo receptor. The latter site allows a genetic fusion of this truncated huEpo-receptor cDNA to a genomic DNA segment encoding the Fc-part of a human IgG molecule. The resulting expression vector pEPORFc codes for a soluble fusion protein consisting of the extracellular region of the huEpo receptor joined to hinge, CH2, and CH3 domains of a human IgG1.

A cell line stably expressing the rhuEpo-receptor Ig fusion protein was established by standard calcium-phosphate transfection of baby hamster kidney cells (BHK 21), using the vector pEPORFc and subsequent methotrexate/G418 double selection as described by Zetlinei1 et al. A production cell line secreting 5 mg/L of rhuEpo-receptor Ig fusion protein was selected from a panel of single cell clones. RhuEpo-receptor Ig fusion protein was afﬁnity-puriﬁed from supernatants using protein-A sepharose (Pharmacia), and characterized by PAGE (Phast System; Pharmacia) and Coomassie staining. The fusion protein was secreted by the production cell line as a dimer.

Epitope exclusion assay. The binding of peroxidase-labeled monoclonal anti-Epo antibodies to rhuEpo was inhibited by nonlabeled homologous or nonhomologous monoclonal anti-Epo antibodies, by the rhuEpo-receptor Ig fusion protein or by huEpo-derived synthetic peptides, or peptides puriﬁed after enzymatic digestion (trypsin, endo Lys C). Microtiter plates were coated with rabbit anti-rhuEpo protein-A-puriﬁed Ig fraction (10 µg/mL) in 50 mM/L phosphate buffer. 10 ng/mL of rhuEpo was bound to the coated microtiter plates for 30 minutes in a water bath at 37°C. After careful washing, 10-fold dilutions of inhibitor (MoAbs or rhuEpo-receptor Ig fusion protein or huEpo-derived peptides; 10 µg/mL to 10 pg/mL, 100 µL/well) were incubated together with peroxidase labeled MoAb.

After 1 hour of incubation, the plates were washed four times. Enzyme activity bound to the wells was determined using tetramethylbenzidine (TMB) as substrate solution. The plates were measured at 495 nm in a Behring ELISA Processor.

Binding assay for receptor-bound rhuEpo. Microtitration plates coated with rabbit antiserum speciﬁc for the CH2 domain of human IgG1 were used to bind the rhuEpo-receptor-Ig fusion protein to solid phase. The receptor molecules were then saturated with rhuEpo and the binding of the peroxidase-labeled MoAbs respectively was determined as described above using TMB as substrate and evaluation at 495 nm in a Behring ELISA Processor.

Characterization of antipeptides. Hybridoma supernatants were screened by ELISA for their capacity to bind to rhuEpo coated to plastic plates or bound to polyclonal anti-Epo antibodies coated to microtitration wells. One set of MoAbs, derived from fusion 89/113 (6 MoAbs), preferentially detected rhuEpo in the more denatured form, directly coated to the plastic wells. However, these MoAbs, coated to a solid phase, rather weakly reacted with soluble peroxidase-labeled rhuEpo. The second set of MoAbs, derived from fusion 89/146 (7 MoAbs), showed good binding to the soluble Epo-POD-conjugate and weaker binding to rhuEpo-coated plastic plates (data not shown). The spleen cell donors of fusion 89/113 had been immunized with antigen preparations containing keyhole limpet hemocyanin (KLH) coupled rhuEpo, whereas those of fusion 89/146 had been immunized with rhuEpo alone, which possibly turned out to be a more native antigen.

RESULTS

Characterization of antibodies. Hybridoma supernatants were screened by ELISA for their capacity to bind to rhuEpo coated to plastic plates or bound to polyclonal anti-Epo antibodies coated to microtitration wells. One set of MoAbs, derived from fusion 89/113 (6 MoAbs), preferentially detected rhuEpo in the more denatured form, directly coated to the plastic wells. However, these MoAbs, coated to a solid phase, rather weakly reacted with soluble peroxidase-labeled rhuEpo. The second set of MoAbs, derived from fusion 89/146 (7 MoAbs), showed good binding to the soluble Epo-POD-conjugate and weaker binding to rhuEpo-coated plastic plates (data not shown). The spleen cell donors of fusion 89/113 had been immunized with antigen preparations containing keyhole limpet hemocyanin (KLH) coupled rhuEpo, whereas those of fusion 89/146 had been immunized with rhuEpo alone, which possibly turned out to be a more native antigen. Antibodies of fusion 89/97 were derived from an immunization with the C-terminal peptide P2 coupled to KLH, followed by boosters with KLH-coupled rhuEpo. These antibodies were P2- and rhuEpo-specific.

The Ig subclasses of the different antibodies determined by immunodiffusion are displayed in Table 1. Furthermore, the binding constants of the antibodies are shown in Table 1. Of all 15 antibodies investigated, 11 displayed signiﬁcant, speciﬁc binding to iodinated rhuEpo in solution under the experimental conditions chosen. The binding constants of MoAbs 146/022 and 146/057 could not be properly evaluated because of persistent excessive scatter of data. This was quite surprising and is so far unexplained because the assay in general yielded binding values of high reproducibility (down to less than 5%, depending on the absolute level of binding). Nevertheless, MoAbs 146/022 and 146/057 were highly speciﬁc for rhuEpo (see also data below in this report), and MoAb 146/057 and its subclone derivatives were very suitable for afﬁnity puriﬁcation of rhuEpo or rhuEpo-variants (data not shown). All of these antibodies were able to bind to deglycosylated rhuEpo in a Western blot, indicating that sugar

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Table 1. Immunizations, Subclasses, and Binding Constants of MoAbs

<table>
<thead>
<tr>
<th>MoAb</th>
<th>Immunization</th>
<th>IgG-Subclass (mouse)</th>
<th>Binding Constant (μmol × 10^-7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>89/113/69 rhuEpo-KLH</td>
<td>IgG1</td>
<td>450.0</td>
<td></td>
</tr>
<tr>
<td>113/029 rhuEpo-KLH</td>
<td>IgG1</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>113/162 rhuEpo-KLH</td>
<td>n.t.</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>113/169 rhuEpo-KLH</td>
<td>IgG1</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>113/71 rhuEpo-KLH</td>
<td>IgG1</td>
<td>35.0</td>
<td></td>
</tr>
<tr>
<td>113/107 rhuEpo-KLH</td>
<td>IgG1</td>
<td>4.2</td>
<td></td>
</tr>
<tr>
<td>89/146/057 rhuEpo</td>
<td>IgG1</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>146/080 rhuEpo</td>
<td>IgG1</td>
<td>58.0</td>
<td></td>
</tr>
<tr>
<td>146/110 rhuEpo</td>
<td>IgG1</td>
<td>37.0</td>
<td></td>
</tr>
<tr>
<td>146/0100 rhuEpo</td>
<td>IgG2a</td>
<td>48.0</td>
<td></td>
</tr>
<tr>
<td>146/0118 rhuEpo</td>
<td>IgG2a</td>
<td>53.0</td>
<td></td>
</tr>
<tr>
<td>146/022 rhuEpo</td>
<td>IgG2b</td>
<td>n.t.</td>
<td></td>
</tr>
<tr>
<td>146/060 rhuEpo</td>
<td>n.t.</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>89/97/034 P2-KLH/rhuEpo-KLH</td>
<td>IgG3b</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>97/175 P2-KLH/rhuEpo-KLH</td>
<td>IgG5b</td>
<td>nd</td>
<td></td>
</tr>
</tbody>
</table>

Epo-receptor-lg — 28.0

The Ig subclasses were determined by double diffusion precipitation. The binding constants were determined by the method of Scatchard. For some antibodies, the binding constant could not be determined (n.t.). In the cases indicated (n.t.) the IgG subclasses were not tested.

residues did not contribute to antibody binding sites (data not shown).

Epitope regions of rhuEpo. To obtain information about the antibody binding determinants on the rhuEpo molecule, 5 of the 14 antibodies were labeled with peroxidase and used in binding inhibition studies. All MoAbs were applied as inhibitors for the binding of the 5 labeled antibodies to huEpo and the inhibitor concentrations necessary for 50% of binding inhibition (ID50) were evaluated, respectively (Table 2). Cross-reacting antibodies recognized the same or adjacent binding sites defining an epitope region. As shown in the table, a single epitope (A, one MoAb) and three distinct epitope regions (B, three MoAbs; C, five MoAbs; and D, six MoAbs) could be defined by different sets of MoAbs.

Interestingly, some antibodies binding to epitope region C could induce enhanced binding of labeled antibody 113/71, which is a member of a group of antibodies binding to epitope region D (Table 2). At high concentrations, MoAb 146/057 binding to epitope region B was able to inhibit the binding of labeled MoAb 113/71, which defines epitope region D. However, conversely, the latter antibody was not able to inhibit labeled MoAb 146/057 (Table 2).

Neutralization of Epo function. To measure the neutralizing activity, MoAbs were used as inhibitors in a rhuEpo-specific erythroid precursor-cell proliferation assay. The erythroid precursors were isolated from a spleen-cell suspension of phenylhydrazine treated mice using Ficoll gradients. The precursor cells were distributed on a microtitration plate and incubated with rhuEpo together with different concentrations of protein A purified MoAbs. MoAbs derived from fusion 89/146 completely inhibited proliferation. However, all but one antibody derived from fusion 89/113 was non-neutralizing. From the inhibition curves, the neutralizing dose of antibody (nmol/mL) at 50% of inhibition (ND50) was determined. The results are given in Table 2. It is obvious from the table that the neutralizing antibodies bound to epitope

Table 2. Epitope Exclusion Assay (ID50 [μg·mL])

<table>
<thead>
<tr>
<th>Epitope Regions</th>
<th>A POD-113/69</th>
<th>B POD-146/057</th>
<th>C POD-146/0100</th>
<th>D POD-113/71</th>
<th>POD-113/107</th>
<th>Neutralization of 0.1 pmol/mL of rhuEpo (ND50) by MoAbs (pmol/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 113/69</td>
<td>10^-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.0</td>
</tr>
<tr>
<td>B 146/057</td>
<td>10^-6-10^-7</td>
<td>10^-2</td>
<td>0.69</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>146/080</td>
<td>10^-3-10^-3</td>
<td>2.45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>146/110</td>
<td>10^-3-10^-4</td>
<td>0.79</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C 146/0100</td>
<td>10^-5</td>
<td>enh 60%</td>
<td>3.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>146/0118</td>
<td>10^-6</td>
<td>enh 30%</td>
<td>5.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>146/022</td>
<td>10^-5</td>
<td>1.53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>146/060</td>
<td>10^-4</td>
<td>enh 50%</td>
<td>4.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>97/175</td>
<td>10^-1</td>
<td>175.0</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>97/034</td>
<td>10^-1</td>
<td>enh 25%</td>
<td>750.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D 113/029</td>
<td></td>
<td></td>
<td></td>
<td>10^-2</td>
<td>10^-2</td>
<td>770.0</td>
</tr>
<tr>
<td>113/162</td>
<td>10^-1-10^-2</td>
<td>10^-2-10^-3</td>
<td>&gt;1,000.0</td>
<td></td>
<td></td>
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<tr>
<td>113/159</td>
<td>10^-1-10^-2</td>
<td>10^-2-10^-3</td>
<td>&gt;1,000.0</td>
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<td></td>
<td></td>
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<tr>
<td>113/71</td>
<td>10^-2</td>
<td>10^-2-10^-3</td>
<td>320.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>113/107</td>
<td>10^-1-10^-2</td>
<td>10^-2-10^-3</td>
<td>&gt;1,000.0</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

According to their cross-reactivity on the rhuEpo molecule different groups of antibodies were listed. Three groups A, B, and C of neutralizing antibodies were identified, whereas group D antibodies were non-neutralizing. For the detection of cross-reactivity peroxidase labeled monoclonal antibodies (POD-MoAbs) were competed for binding to antibody bound rhuEpo by nonlabeled MoAbs. The values for 50% of inhibition are shown. Group C antibodies induced enhanced binding of POD 113/71. The factor of enhancement of 100% binding is shown for a concentration of 10 g of antibody per milliliter of solution. Neutralizing activity was assayed according to Krystal (1986, see Materials and Methods). The doses of 50% of neutralization (ND50) are displayed for each antibody. The results correspond to the mean values of triplicate samples, respectively.
regions A, B, or C, respectively, whereas all non-neutralizing antibodies bound to epitope region D.

The fact that we identified three different binding regions for different sets of rhuEpo neutralizing antibodies raised the question if any of these regions could cover the rhuEpo-receptor binding site. To investigate this, a rhuEpo receptor binding assay was established.

A soluble rhuEpo-receptor molecule was developed by fusion of the extracellular region of the human Epo-receptor to the hinge, CH2-domains, and CH3-domains of a human IgG1 molecule. This soluble receptor protein binds rhuEpo specifically and with high affinity (Table 1) and allowed us to carry out rhuEpo receptor binding-competition analysis. In the particular assay, the rhuEpo-receptor–Fc molecule was used as inhibitor of peroxidase-labeled antibodies binding to rhuEpo, which had been bound to solid phase by rabbit anti-rhuEpo antibodies. Only neutralizing MoAbs 146/080 and 146/057, defining epitope region B, were competed by the receptor molecule (Fig 1).

On the other hand, neither neutralizing antibody 146/0100 (against epitope region C) nor neutralizing antibody 113/69 (against epitope region A) could be inhibited by the rhuEpo-receptor Ig fusion protein (Fig 1). This indicated that the epitopes of these antibodies are different from determinants contributed by the receptor binding site of the rhuEpo molecule. However, when these MoAbs were reacted against receptor-bound rhuEpo, they were unable to bind, although they displayed good binding to polyclonal-antibody–bound rhuEpo (Fig 2).

Epitope mapping. To map sequential epitopes defined by the antibodies or the rhuEpo-receptor Fc fusion protein, 20 tryptic, 9 endo-Lys-C, and 5 synthetic peptides described previously and 3 additional synthetic peptides were purified and used as inhibitors in an ELISA. In this assay, Epo was bound to a polyclonal rabbit-anti-huEpo Ig fraction coated to microtiter plates. The synthetic peptide B12, covering amino acids 111-127 of the huEpo molecule, was able to act as competitor of the peroxidase-labeled MoAb 113-107, defining epitope region D (data not shown). A different synthetic peptide, covering the N-terminus (amino acids 1 to 17) competed for the binding of peroxidase-labeled MoAb 146-050, binding to epitope region C. The epitope of this antibody is possibly limited to the first 10 N-terminal amino acids, because a different peptide (A2), starting with amino acid 11, did not interact with this antibody. All the other peptides were unable to compete antibody binding (data not shown).

In a different assay, pools of trypsin or endo-Lys-C–derived peptides were used as inhibitors for the binding of the peroxidase-labeled rhuEpo to the rhuEpo-receptor Fc fusion protein. No effective competitor could be identified, although 1,000-fold excess of inhibitor was used (data not shown).

In a further approach we used the C-terminal peptide P2 as antigen for immunizations to obtain antibodies recognizing sequential epitopes. It was not possible to obtain rhuEpo-specific high-affinity mouse antibodies, although the synthetic peptide P2 had proven to be suitable for the induction of well-neutralizing polyclonal rabbit antibodies. Only 2 antibodies (97/034 and 97/175) out of 25 peptide P2-specific antibodies could be obtained, which reacted with peptide P2 and, in addition, with the whole molecule of rhuEpo. Although both of them reacted with epitope region C, only antibody 97/175 could neutralize the rhuEpo function in the in vitro assay. However, when compared with the rhuEpo-induced neutralizing antibodies, a 100-fold to 1,000-fold

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Fig 1. Competition of peroxidase-labeled MoAbs binding to solid-phase–bound rhuEpo through rhuEpo-receptor Fc fusion protein. The fusion protein and homologous antibodies were used as competitors. The percentage of binding was plotted against the competitor concentration. Optical density (OD) was measured at 495 nm. (□) No competitor; (◼) + homologous MoAb; (◼) + rhuEpo-receptor.

Fig 2. Direct binding of peroxidase-labeled MoAbs to rhuEpo-receptor Ig fusion protein or polyclonal anti-rhuEpo antibody-bound rhuEpo. Microtitration plates coated with rabbit antihuman IgG-Fc and with rhuEpo-receptor Ig or with polyclonal anti-rhuEpo were incubated with various dilutions of peroxidase-labeled anti-rhuEpo MoAbs. For each peroxidase conjugate, the binding of the same dilution was compared for antibody- and receptor-bound (■) rhuEpo. Optical density was measured at 495 nm. The binding capacities for rhuEpo-receptor Ig–bound rhuEpo and for polyclonal antibody–bound rhuEpo were compared.
higher concentration was necessary for 50% inhibition of the biological activity (Table 2).

**DISCUSSION**

This study presents sets of neutralizing and non-neutralizing MoAbs directed against rhuEpo, which were selected from three fusions: 89/97, 89/113, and 89/146. Binding inhibition studies with these MoAbs showed that the neutralization-mediating epitopes of native EPO were concentrated in three spatially distinct epitope regions: A, B, and C. Antibodies derived from fusion 89/146 preferentially bound to epitope regions B and C, and antibodies from fusion 89/97 interacted only with epitope region C. MoAb 113-69, the only neutralizing antibody of fusion 89/113, was not cross-inhibited by the neutralizing antibodies of fusion 89/146 or by the non-neutralizing antibodies derived from the fusion 89/113 and, therefore, defined an isolated epitope (epitope A). The non-neutralizing MoAbs of fusion 89/113 defined a separate epitope region (D) which did not show interaction with the neutralizing antibodies (113/69 and antibodies of fusion 89/146). Thus, it seems likely that the murine immune response is restricted to four antigenic domains of the rhuEpo molecule.

MoAbs 146/057 and 146/080, binding to epitope region B, were found to compete with the soluble rhuEpo-receptor Fc fusion protein for its binding site on the rhuEpo molecule. From these findings, we concluded that the epitopes of region B contributed to the three-dimensional structure of the receptor binding site and we suggest that they are also involved in receptor binding on the cell surface.

Evidence for adjacently located epitopes of different epitope regions was provided by MoAb 146/057 (epitope region B), which could compete for binding of labeled MoAb 113/71 (epitope region D). In the in vitro proliferation assay, MoAb 113/71 was the only group D antibody able to weakly neutralize the function of rhuEpo, supporting the suggestion that this antibody might bind to a determinant connecting the two epitope regions B and D, adjacent to the receptor binding site.

The non-neutralizing MoAb 113/107, defining epitope region D specifically interacted with synthetic peptide B12, covering the region of amino acids 111 to 127 of the huEpo sequence. This result corroborates our earlier finding with rabbit antibodies, that we were unable to find a site of neutralization in the central part of the huEpo primary sequence.7

However, Sytkowski et al6 and Feldman et al8 provided some evidence, that the central part of the Epo molecule (amino acids 99 to 129) might be involved in biological activity because they found neutralizing antibodies binding to synthetic peptides covering that region. Additionally, they could eliminate biological activity by creating deletion-replacement mutations in that region (amino acids 99 to 110).9

As the cross-reaction of our group B and group D antibodies (see above) suggests that the two regions could be spatially adjacent, the central region D could be involved, at least, in stabilization of the receptor binding site and it is possible that this region too contains some receptor binding determinants (see data of MoAb 113/71, above, and Chern et al15).

It was not possible to identify a peptide sequence carrying the binding determinants of the rhuEpo-receptor or the group B antibodies. Similar results with rhuEpo-induced mouse antibodies were also obtained by Wognum et al11 and D’Andrea et al,10 who found neutralizing MoAbs against conformational epitopes. Furthermore, neutralizing group B antibody 146/057 probably detected a rather variable conformational epitope, possibly provoking the unexpected binding characteristics of this antibody. Our results support the data of others,6 providing evidence that the receptor binding site of rhuEpo might be exclusively composed of conformational determinants defined by multiple peptide components rather than by a single peptide fragment. However, it should be emphasized that only a limited number of possible peptide structures has been tested in our study.

D’Andrea et al10 found a non-neutralizing rhuEpo-induced MoAb reactive with the endo-Lys-C–derived 10 residues N-terminal peptide. In a former study, Sue and Sytkowski15 had been successful in developing a urinary human Epo-specific MoAb using the 26 residue N-terminal peptide of huEpo as antigen. Again, this N-terminus-specific antibody was non-neutralizing. In contrast, we found a neutralizing group C antibody (146/050) interacting with the first 10 N-terminal amino acids. Therefore, it seems likely that neutralizing and non-neutralizing MoAbs can be directed against one and the same linear N-terminal epitope of epitope region C. Similar results were observed for epitope region D (see above).6,7,18

Furthermore, using the carboxyl-terminal peptide P2 of huEpo as antigen we found two rhuEpo-specific antibodies. These P2/rhuEpo-specific antibodies also interacted with epitope region C (MoAbs 97/34 and 97/175) indicating that the C-terminus, too, could contribute to the formation of this epitope region. This suggestion becomes more plausible by the fact that both the N-terminus and C-terminus are linked by a disulphide bond, guaranteeing the stability of the tertiary structure, which is essential for biologic activity.

One of the peptide-specific MoAbs (97/175) was able to significantly neutralize the function of rhuEpo, although only at high antibody concentrations (80% inhibition at 10 μg of inhibitor MoAb per mL). This weak interaction probably was a result of the low affinity of this P-2-KLH–induced antibody. Although this result supported a possible contribution of the C-terminus to the receptor binding site,7 further experiments based on rhuEpo-receptor Fc fusion protein gave evidence that this conclusion might be wrong. Neither labeled P2-specific neutralizing MoAb 97/175, nor labeled neutralizing antibody 146/0100, both binding to epitope region C, competed with the rhuEpo-receptor-Fc fusion protein for binding to rhuEpo. From this it became evident that a possible contribution of the carboxyl-terminal peptide to the receptor binding site of rhuEpo was only of minor importance, although these antibodies were able to neutralize. Similarly, neutralizing group A antibody 113/69 was not competed by rhuEpo-receptor Fc fusion protein and, therefore, was not specific for the receptor binding site.

Although group A and C antibodies were able to bind to polyconal antibody–bound rhuEpo in the presence of soluble-receptor Fc as a competitor, they were not able to bind to receptor-bound rhuEpo. This could indicate that a con-
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formational change of rhuEpo is induced by the receptor, suggesting that rhuEpo complexed with the receptor does not present group A or group C epitopes and, therefore, cannot be competed by these antibodies. Alternatively, it could be suggested that rhuEpo-neutralizing group A and group C antibodies can transmute soluble rhuEpo to a nonfunctional molecule. However, it cannot be ruled out that steric hindrance could be the causa sine qua non of rhuEpo neutralization by MoAbs binding to epitopes adjacent to the receptor binding site.

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